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The Evolution of Energy-Transducing Systems. Studies with an Extremely Halophilic Archaebacterium.

Final Report

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[1]The NASA Technical Officer for this grant is Dr. L.I.Hochstein, NASA Ames Research Center, Moffett Field, CA 94035

[2]Abbreviations: DCCD, dicyclohexylcarbodiimide; NEM, N-ethylmaleimide; PVDF, polyvinylidene difluoride

Summary of research results:

The F-type ATPases are found in remarkably similar versions in the energy-transducing membranes of eubacteria, chloroplasts and mitochondria (1). Thus, it is likely that they have originated early in the evolution of life, which is consistent with their function as key enzymes of cellular metabolism. The archaebacteria are a group of microorganisms which, as shown by molecular sequencing and biochemical data, have diverged early from the main line of prokaryotic evolution (2). From studies of members of all three major groups of archaebacteria - the halophiles, methanogens and thermoacidophiles - it emerged that they possess a membrane ATPase which differs from the F-ATPases. The goal of this project was a comparison of the ATPase from the halophilic archaebacterium *Halobacterium saccharovorum* with the well-characterized F-type ATPases on the molecular level. Amino acid sequences of critical regions of the enzyme were to be determined, as well as immunoreactions of single subunits in the search for common epitopes. The results were expected to allow a decision about the nature of archaebacterial ATPases, their classification as one of the known or, alternatively, novel enzyme complexes, and possibly deduction of events during the early evolution of energy-transducing systems.
Since both ATPase enzymes are large multi-subunit complexes (molecular mass of approximately 540 kDa in the case of the F-ATPase from the eubacterium *Escherichia coli*), careful consideration was given to the choice of the peptide fragments which were to be sequenced. Previous studies in Dr. Hochstein's laboratory had indicated a preferential incorporation of an inhibitor of F-type ATPases, DCCD[2)], into the second largest subunit (subunit II) of the ATPase from *H. saccharovorum* (3). The conditions for incorporation of this inhibitor were similar to those which were used for DCCD-labeling of the beta subunit of the ATPase of bacteria and mitochondria. Most importantly, activity of the halobacterial ATPase enzyme was reduced upon reaction with the inhibitor. The DCCD-binding peptide from several F-type ATPases had been sequenced and was found to be highly conserved between different species. Not surprisingly, this peptide is in a region of the beta subunit of the enzyme complex which is thought to be involved in catalysis.
Thus, with the information available at the start of the project it seemed feasible to locate a similar peptide in the halobacterial ATPase which had

DCCD covalently bound, to determine its amino acid sequence and to search for homologies (or the lack thereof) to the known F-ATPases.
While the labeling of large batches of purified halobacterial ATPase with DCCD was accomplished readily, the workup of the modified enzyme presented numerous technical problems (described below), most of which can probably be ascribed to the extremely acidic nature of halobacterial proteins. Several peptides were isolated, though in low yield, from labeled subunit II and their sequences determined as follows:

H. saccharovorum , 1. peptide:	NTLVRGQKLPIF
H. salinarium (Ref.4):	NTLVRGQKLPIF
H. saccharovorum , 2. peptide:	YTDLAQLYERAG
H. salinarium (Ref.4):	YTDLAQLYERAG

From these data it appeared that there might be considerable similarity of the ATPase from *H. saccharovorum* to those of other archaebacteria, e.g. the halophile *H. salinarium* (4). The actual binding site (or sites) of DCCD could not yet be pinpointed to single amino acid residues, but the approximate location with respect to fragments obtained by cyanogen bromide cleavage was possible. Two peptides are still to be sequenced which should shed light on this question
The inhibition of the halobacterial enzyme by DCCD was not relieved by the presence of cations or nucleotides, which is in contrast to the behaviour of F-type ATPases. Thus, it appeared that the mechanism of inhibition of the halobacterial enzyme must be different. Double-labeling studies with DCCD and NEM[2)] suggested that the interaction of the large subunits may be disrupted by the incorporation of DCCD and that this effect might account for the inactivation of the enzyme.
From the DCCD and other labeling studies as well as from immunological crossreaction with various beta subunits, it was deduced that subunit II of the halobacterial enzyme might be the catalytic subunit. However, new information from archaebacterial and eukaryotic ATPases suggested otherwise. Thus, extensive homologies between the DNA sequences of the major ATPase subunits from archaebacteria with the vacuolar-type ATPases were reported (5,6). Vacuolar (V-type) ATPases are found in the endomembrane system of eukaryotes and are, like the F-ATPases, large multisubunit complexes (7). The catalytic site is located in the largest subunit (subunit A). Thus, a comparison of the halobacterial ATPase with vacuolar ATPases seemed to be called for and was greatly facilitated by the interest in collaborative research of one of the pioneering groups in the

vacuolar ATPase field (B. and E. Bowman at the University of Santa Cruz). The experiments yielded immediate and rather clear-cut results: crossreaction of subunit I of the halobacterial ATPase with an antiserum raised against subunit A of the vacuolar ATPase from *Neurospora crassa*; recognition of only one peptide of the size of subunit I in halobacterial membranes by the same antiserum; inhibition of the halobacterial ATPase by NEM, as shown for V-ATPases; demonstration of the incorporation of NEM into subunit I at a cysteinyl residue; reduction of that incorporation by nucleotides and concomitant reduction of inhibition. In addition, it could be shown that the antiserum against subunit A recognized a polypeptide of similar mobility as subunit I in various halophilic isolates, some of them representing novel species. An extension of this work to the ATPase of another archaebacterium, *Sulfolobus solfataricus* , which has been studied in the laboratory of Dr. Hochstein, suggested the similarity of some features of that enzyme to the vacuolar ATPases as well.
The conclusions drawn from these results were the existence of similarities between archaebacterial and vacuolar ATPases in structure and mechanism, and were published in several papers (8-11). However, many intriguing questions have not been answered, e.g. it is not clear what the function of the archaebacterial ATPases is. The function of vacuolar ATPases is the establishment of a proton gradient by the hydrolysis of ATP, but they do not synthesize ATP. Archaebacterial ATPases are presumed to operate, besides in the hydrolytic mode, also in the ATP synthesizing mode, but there is no experimental evidence for this as yet.

The Cooperative Agreement is expected to be continued under the title "The Evolution of Energy-Transducing Systems. Studies with Archaebacteria". The focus of the anticipated research will be somewhat shifted, though. The experience gained through the attempts of isolating and sequencing the DCCD-binding peptides of the halobacterial ATPase will facilitate the isolation of the NEM binding peptide from subunit I. Thus, a comparison of the sequence around the NEM binding site should allow the identification of the nucleotide binding site of the halobacterial ATPase. The bulk of the proposed research under the new agreement will, however, be the search for the membrane sector of the halobacterial ATPase. Such a sector might be found by labeling studies; it will then be most interesting to establish the association with the known halobacterial ATPase or perhaps with a different enzyme. Evidence that there might exist a so far unknown ATPase

in the halobacterial membranes comes from recent studies on ATP synthesis by Dr. Hochstein (12).

Technical description of experiments and procedures

Although halobacteria have been studied in detail for over 30 years and the recognition of this group as members of the archaebacteria dates back to 1977, comparatively few studies deal with their proteins on a molecular level. This reflects methodological problems inherent in the requirement of many halobacterial enzymes for high concentrations of salt, often in the range of 4 M NaCl, which renders many commonly used methods for purification and characterization of proteins useless.
A brief account of the experiences with the preparation of peptides from the ATPase from *H. saccharovorum* as well as with immunological studies, which were conducted during the course of the project, will be given below.

1. Purification of the halobacterial ATPase enzyme on a large scale:
These experiments presented the least problems; published procedures could be readily scaled up from starting material of 10 l to 40 or 80 l of bacterial culture. The chromatography on phenylsepharose was slightly modified by eluting the enzyme into a 4 M ammoniumsulfate/Tris buffer under stirring to maintain a stable environment for the enzyme.

2. Labeling of the ATPase with ^{14}C-DCCD:
Problems were encountered when attempting to separate enzyme from unbound label, since a significant portion of subunits II was lost following chromatography on several resins, probably due to strong adsorptive forces. This was in contrast to the F1 ATPase of *E.coli* , which is recovered intact on filtration through Sephadex G50 following labeling with DCCD. Finally a procedure of concentrating and "washing" the enzyme with buffer in centricon microconcentrators was adopted for removal of DCCD from the halobacterial enzyme.

3. Separation of the subunits following SDS polyacrylamide gel electrophoresis:
Individual subunits were excised from SDS gels and electroeluted from the gel pieces; recovery was greater than 85 % for subunit II. When electroelution was conducted for more than 3.5 hours, some degradation of

the subunits was observed.

4. Enzymatic and chemical cleavage:
Enzymatic cleavage of labeled and electroeluted subunit II was performed with V8 protease, chymotrypsin and clostripain. None of these enzymes produced a complete cleavage, even after repeated treatments up to a total of 3 days. However, chymotrypsin appeared to cleave at least 60 - 70 % of the starting material.
Chemical cleavage with cyanogen bromide was used later in the project. While no uncleaved subunit II remained after treatment with CNBr, several fragments appeared with molecular weights of about 10-14 kDa. The amino acid sequence of subunit II is not known; however, if it is similar to that of *H. salinarium* (see Ref.4), these peptides would represent incompletely cleaved material.

5. Separation of peptides on a reverse phase column:
This procedure presented the severest problems. While initial separation of peptides from the reaction mixture could be achieved with an acetonitrile gradient, re-chromatography led to losses of up to 99 %. Similar difficulties, particularly with acidic peptides, have been widely described in the literature on peptide separation by HPLC. Losses of halobacterial peptides were in part due to adsorption to glass ware; some improved recovery was achieved after switching to plastic tubes and injection syringes. Another problem was the appearance of numerous radioactive peaks following separation. Most likely this was due to incomplete cleavage (see above) by proteases; however, there could also be multiple elution of peptides. Due to the low recoveries after re-chromatography this potentially important point could not be verified. One peptide obtained by reverse phase chromatography was sequenced; the amount was very low, though, and thus the sequence remained speculative. However, it was unlike any sequence from the beta subunits of F-ATPases.

7. Separation of CNBr fragments by SDS gel electrophoresis and subsequent electroelution:
Tris/tricine gels were used for the separation of small peptides. The newly developed method of staining proteins with cupric chloride worked well with the fragments of subunit II. However, the procedure of destaining with EDTA eluted most of the peptide material from the gel slices. Incomplete

destaining, on the other hand, interfered with subsequent electroelution. However, it was possible to obtain partial sequences from 2 peptides prepared this way (sequences are given in Summary).

8. Blotting of CNBr fragments on PVDF[2]:
This method has been widely used since its invention (Ref.13) for direct sequencing of the blotted peptides. The standard buffer (10 mM CAPS, pH 11, 10 % methanol) did not promote efficient binding of the halobacterial peptides to the PVDF membrane, although it did so for several CNBr fragments of myoglobin. Several other transfer buffers were tried; most efficiently was the Towbin buffer used for Western blots (Ref.14). However, this buffer contains glycine and Tris, which might interfere with sequencing. At this time, two CNBr fragments of subunit II, blotted with Towbin buffer on PVDF, have been sent out for sequencing.

9. Western blots of halobacterial ATPase subunits:
Very efficient binding of both major subunits to nitrocellulose membranes was obtained. Crossreactions of antisera to subunits I and II with the major subunits of the vacuolar ATPase could be demonstrated and, interestingly, also to alpha and beta subunits of the *E. coli* F-ATPase (to be presented as poster at ASM conference Nov. 1992)

References

1. Pederson,P.L. and Carafoli,E. (1987) Ion motive ATPases. I.Ubiquity, properties and significance to cell function. Trends Biochem.Sci.12, 146-150.

2. Woese, C.R. (1987) Bacterial Evolution. Microbiol. Rev. 51, 221-271.

3. Kristjansson,H., Sadler,M.H. and Hochstein,L.I., (1986) Halobacterial adenosine triphosphatases and the adenosine triphosphatase from Halobacterium saccharovorum. FEMS Microbiol. Rev. 39,151-157.

4. Ihara,K. and Mukohata,Y. (1991) The ATP synthase of Halobacterium salinarium (halobium) is an archaebacterial type as revealed from the amino acid sequences of its two major subunits. Arch. Biochem.Biophys.286, 111-116.

5. Denda,K., Konishi,J., Oshima,T., Date,T., and Yoshida,M. (1988a) The membrane-associated ATPase from Sulfolobus acidocaldarius is distantly related to F_1-ATPase as assessed from the primary structure of its a-subunit. J.Biol. Chem.263, 6012-6015. Denda,K., Konishi,J., Oshima,T., Date,T., and Yoshida,M. (1988b) Molecular cloning of the b-subunit of a possible

non-F_0F_1 type ATP synthase from the acidothermophilic archaebacterium, Sulfolobus acidocaldarius. J.Biol. Chem. 263, 17251-17254.

6.Inatomi,K., Eya,S., Maeda,M, and Futai,M. (1989) Amino acid sequences of the a and b subunits of Methanosarcina barkeri ATPase deduced from cloned genes. J.Biol.Chem.264,10954-10959.

7. Forgac, M. (1989) Structure and Function of vacuolar class of ATP-driven proton pumps. Physiol.Rev. 69, 765-796.

8. Stan-Lotter,H., Bowman,E.J., and Hochstein,L.I. (1991) Relationship of the membrane ATPase from Halobacterium saccharovorum to vacuolar ATPases. Arch.Biochem. Biophys. 284, 116-119.

9. Sulzner,M., Stan-Lotter,H. and Hochstein,L.I. (1992) Nucleotide-protectable labeling of sufhydryl groups in subunit I of the ATPase from Halobacterium saccharovorum. Arch.Biochem. Biophys. 296, 347-349.

10. Hochstein,L.I. and Stan-Lotter,H. (1992) Purification and properties of an ATPase from Sulfolobus solfataricus. Arch.Biochem.Biophys. 295, 153-160.

11. Stan-Lotter,H., Sulzner, M., Egelseer, E., Norton, C.F. and Hochstein, L.I. (1992) Comparison of membrane ATPases from extreme halophiles isolated from ancient salt deposits. Origins of Life (in press).

12. Hochstein, L.I. (1992). ATP Synthesis in Halobacterium saccharovorum: Evidence that synthesis may be catalyzed by an FoF1 ATP synthase. FEMS Microbiol. (in press)

13. Matsudeira,P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J.Biol.Chem. 262, 10035-10038.

14. Towbin,H., Staehelin and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc.Natl. Acad.Sci.USA 76, 4350-4354.

Publications during tenure of NCC2-578

Papers

Stan-Lotter,H., Sulzner, M., Egelseer, E., Norton, C.F. and Hochstein, L.I. (1992) Comparison of membrane ATPases from extreme halophiles isolated from ancient salt deposits. Origins of Life (in press).

Denner, E. and Stan-Lotter, H. (1992) On the isolation of halophilic

microorganisms from salt deposits of great geological age. NASA Conference Proceedings (in press).
Sulzner,M., Stan-Lotter,H. and Hochstein,L.I. (1992) Nucleotide-protectable labeling of sufhydryl groups in subunit I of the ATPase from Halobacterium saccharovorum. Arch.Biochem. Biophys. 296, 347-349.

Hochstein,L.I. and Stan-Lotter,H. (1992) Purification and properties of an ATPase from Sulfolobus solfataricus. Arch.Biochem.Biophys. 295, 153-160.

Stan-Lotter,H., Bowman,E.J., and Hochstein,L.I. (1991) Relationship of the membrane ATPase from Halobacterium saccharovorum to vacuolar ATPases. Arch.Biochem. Biophys. 284, 116-119.

Stan-Lotter,H., Lang, F.J. Jr. and Hochstein, L.I. (1989) Electrophoresis and isoelectric focusing of whole cell and membrane proteins from the extremely halophilic archaebacteria. Appl.Theor. Electrophor.1,147-153

Abstracts

Stan-Lotter, H., and Hochstein, L.I. Immunological approaches to the evolution of halobacterial ATPases. ASM Conference on Halophilic Bacteria, Williamsburg VA,1992.

Denner,E., Grant, W.D. and Stan-Lotter,H. A novel halophilic coccoidal isolate from salt sediments. ASM Conference on Halophilic Bacteria, Williamsburg VA,1992.

Stan-Lotter,H., Lang,F.J., Richter,M., Neuninger, C. and Hochstein, L.I. (1992) Rapid identification of halobacterial species by protein gel electrophoresis. Abstr. I-34, ASM Meeting, New Orleans

Stan-Lotter,H. (1991) Comparison of proteins from halophilic archaebacteria and microbial isolates from ancient salt deposits. Abstr. Eur.Geophys.Soc. XVI Ass. Wiesbaden, PS 11.

Sulzner,M., Stan-Lotter, H., and Hochstein, L.I. (1991). Nucleotide protectable labeling of sulfhydryl groups in subunit I of the ATPase from Halobacterium saccharovorum. Abstr.K-73, ASM Meeting, Dallas.

Stan-Lotter, H., Sulzner,M. and Hochstein, L.I. (1991). Functional molecular mass and nucleotide protectable labeling of the membrane ATPase from Halobacterium saccharovorum. Gordon Conference, Protons and Membrane Reactions, Ventura.

Sulzner,M., Egelseer,E. and Stan-Lotter,H. (1991) Analysis of archaebacterial and eubacterial ATPases by radiation inactivation and immunoreactions. Abstr.P 16 VAAM Meeting, Freiburg. Bioforum 14, 39.

Sulzner,M. and Stan-Lotter,H. (1990) Functional mass of the membrane ATPase from the archaebacterium Halobacterium saccharovorum. Abstr.VAAM Meeting, Berlin,P 57.

Hochstein, L.I. and Stan-Lotter,H. (1990) The ATPase from Halobacterium saccharovorum is a chimeric enzyme. Abstr.Int.Conf.Archaebacteria, Osaka, Nov.1990, pg.13.

Stan-Lotter,H. Lang, F.J.Jr. and Hochstein,L.I. (1989) Isoelectric focusing of proteins from extremely halophilic archaebacteria. Abstr. FEMS NATO Workshop Adv.Res., Alicante, Spain.

Invited seminars:

May 26, 1989, Dept. of Biology, University of California at Santa Cruz; title: Which type of ATPase is present in the Halobacteria?

January 26, 1990, Dept. of Biology, University of Nevada at Las Vegas; title: Membrane proteins from the extremely halophilic archaebacteria.

December 4, 1990, Institute of Theoretical Chemistry, University of Vienna, Austria; title: ATPase enzyme from halobacteria.

March 18, 1992, Austrian Association for the Advancement of Natural Sciences, Vienna; title: Archaebacteria and the search for extraterrestrial life (in German)